

Article



# Species-Specific Nitrogen Resorption Efficiency in *Quercus* mongolica and Acer mono in Response to Elevated CO<sub>2</sub> and Soil N Deficiency

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Abstract: To test the effects of elevated CO2 and soil N deficiency on N resorption efficiency (NRE) from senescing leaves in two non-N2-fixing deciduous broadleaved tree species, Japanese oak (Quercus mongolica var. grosseserrata Blume) and Painted maple (Acer mono Maxim. var. glabrum (Lév. Et Van't.) Hara), potted seedlings were grown in a natural daylight phytotron with either ambient or elevated CO<sub>2</sub> conditions (36 Pa and 72 Pa CO<sub>2</sub>) and with two levels of N (52.5 and 5.25 mg N pot<sup>-1</sup> week<sup>-1</sup> for high N and low N, respectively). We examined the N content ( $N_{mass}$ ) of mature and senescent leaves, as well as photosynthesis and the growth of plants, and calculated both the mass-based NRE ( $NRE_{mass}$ ) and leaf area-based NRE ( $NRE_{area}$ ). In both species, the  $N_{mass}$ of mature leaves decreased with high CO2 and low N, whereas the leaf mass per area (LMA) increased under elevated CO2, regardless of N treatments. In Q. mongolica, both the maximum rate of carboxylation ( $V_{cmax}$ ) and the maximum electron transport rate ( $J_{max}$ ) were reduced by elevated CO<sub>2</sub> and low N, but  $V_{cmax}$  exhibited an interactive effect of N and CO<sub>2</sub> treatments. However, in A. mono, both the  $V_{\rm cmax}$  and  $J_{\rm max}$  decreased under elevated CO<sub>2</sub>, regardless of N treatments. The partitioning of N for the photosynthetic function within leaves was also significantly decreased by elevated CO<sub>2</sub> in both species and increased under low N in A. mono. The N<sub>mass</sub> of senesced leaves decreased under low N in both species and exhibited an increase (Q. mongolica) or no effect (A. mono) by elevated CO<sub>2</sub>. The NREarea of Q. mongolica was affected by CO2 and N treatments, with a decrease under elevated CO<sub>2</sub> compared to ambient CO<sub>2</sub> and under low N compared to high N. The NRE<sub>area</sub> of A. mono was also affected by CO<sub>2</sub> and N treatments and decreased under elevated CO<sub>2</sub>; however, unlike in the case of Q. mongolica, it increased under low N. We speculate that these interspecific differences in the responses of leaf N allocation, indicated by the photosynthetic ( $V_{cmax}$  and  $J_{max}$ ) and morphological (LMA) responses to elevated CO<sub>2</sub>, may have affected the NRE during defoliation under high CO<sub>2</sub> and soil N-deficient conditions.

Keywords: leaf litter; leaf mass per area; photosynthetic downregulation

### 1. Introduction

The increasing atmospheric CO<sub>2</sub> concentration is a crucial factor influencing global climate change [1,2]. Projections for atmospheric CO<sub>2</sub> concentrations are provided by distinct representative concentration pathways (RCPs). According to the projected trajectories, the CO<sub>2</sub> concentration in 2100 may range from 421 (RCP2.6) to 936 ppm (RCP8.5, a scenario with very high greenhouse gas emissions; [2,3]). Elevated CO<sub>2</sub> stimulates photosynthesis, which can increase forest net primary production but, at longer timescales, may not necessarily increase plant biomass. Biomass increase depends on nutrient availability, rising temperatures, heat stress, and variation in precipitation and plant water availability [4]. The nutrient limitation, especially N, will also determine ecosystem responses



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**Copyright:** © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). to elevated  $CO_2$  [1,5–9]. A recent study reported that the degree to which N limitation diminished the effects of  $CO_2$  on plant growth varied among experiments, depending on the age of forests and distinct nutrient strategies, for example, those involving microbial associations [4,10,11].

N is absorbed into the soil mainly through leaf litter. The initial N content or C/N ratio of leaf litter affects its rate of decomposition [12] and influences the growth of trees through the nutrients supplied to the soil. Elevated atmospheric CO<sub>2</sub> can affect the quality of leaf litter and even the rate of its decomposition [13–15]. Indeed, the green leaves of many tree species show decreased N concentrations under elevated CO<sub>2</sub> [16,17]. Strain and Bazzaz [18] proposed the litter quality hypothesis, i.e., that a decrease in the N concentration of green leaves under high CO<sub>2</sub> causes a decrease in the N concentration of fallen leaves and a slower decomposition rate. However, there is no consistent view on how high CO<sub>2</sub> affects the N concentration of leaf litter [13,19–23] and its decomposition rate [24–26].

Leaf litter N levels depend on the characteristics of green leaves and nutrient resorption during leaf senescence. N resorption from senescing leaves is the primary mechanism by which plants conserve nutrients [27,28]. N resorption efficiency (NRE), which is often expressed as the fraction of N resorbed at senescence relative to green leaf N [29], can be influenced by numerous factors, including soil N availability [30,31], N additions [32], climate zone (mean annual precipitation and latitude), plant age [33–35], plant functional types [36,37], and the balance between N and P availability [38].

Under high CO<sub>2</sub>, the ratio of soluble N, which is easily reabsorbed, has been reported to decrease in relation to the ratio of structural N, which is used for cell walls and is less easily reabsorbed; thus, NRE may decrease through a decrease in the ratio of leaf soluble N to structural N [16,39–41]. However, it has also been reported that NRE did not change [22,42–45] or exhibited an increase [22,23,43,46,47] under elevated CO<sub>2</sub>. Unfortunately, data on how elevated CO<sub>2</sub> affects NRE are scant, relative to data on how it affects photosynthetic activities in green leaves. Therefore, a consensus has yet to be reached on how high CO<sub>2</sub> affects the NRE of deciduous tree species [21].

N that has been allocated to metabolic proteins, many of which are enzymes associated with photosynthesis [48], is easily degraded and reabsorbed during leaf senescence [49,50]. The downregulation of photosynthesis under high CO<sub>2</sub> levels often leads to decreases in the maximum CO<sub>2</sub> assimilation rate ( $V_{cmax}$ ), which represents the amount or activity of Rubisco, the most abundant plant protein [51–54]. In addition, the leaf mass per area (LMA), which correlates with the amount of N allocated to structural proteins [55], often increases under high CO<sub>2</sub> [51]. Based on the above idea that NRE may be reduced with a decrease in the ratio of soluble N to structural N in leaves, the response of NRE to high CO<sub>2</sub> may be related to this photosynthetic response and the response of LMA in each species.

The sink–source balance, that is, whether the sink capacities for the additional photosynthate are adequate or insufficient for each plant, is one explanation for photosynthetic downregulation under high CO<sub>2</sub> [56]. N<sub>2</sub> fixers, such as *Alnus* species with indeterminate growth, are largely independent of soil N, and their photosynthetic activity and growth responses to increased CO<sub>2</sub> may be more direct than those of non-N<sub>2</sub> fixers [52,57,58]. In previous studies of Manchurian alder (*Alnus hirsuta* Turcz.), distinct photosynthetic downregulation and a marked increase in LMA were not observed under high-CO<sub>2</sub> treatment [59,60]. Moreover, an interactive effect of CO<sub>2</sub> and N treatments on the area-based NRE of *A. hirsuta* was also observed, which decreased under elevated CO<sub>2</sub>, with only N-deficient soil [59], and only a small change in the NRE of *A. hirsuta* under high CO<sub>2</sub> levels. The response of photosynthetic activity, LMA, and NRE to high CO<sub>2</sub> levels in *A. hirsuta* may support the idea mentioned above.

The two non-N<sub>2</sub>-fixing deciduous broadleaved tree species Japanese oak (*Quercus mongolica* var. *grosseserrata* Blume) and Painted maple (*Acer mono* Maxim. var. *glabrum* (Lév. Et Van't.) Hara), which are representative of mid- and late-successional species, respectively, from the boreal forests of Northeast Asia, express flushing-type phenology (determinate growth). Since species with determinate growth are prone to downregulate

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photosynthesis due to the sink limitation for photosynthates [56], these two species may clearly downregulate photosynthesis, especially in N-deficient soil conditions, compared to *A. hirsuta*. As a result, the NRE may be more reduced at high CO<sub>2</sub> in *Q. mongolica* and *A. mono* than in *A. hirsuta*.

We hypothesized that NRE would be reduced in Q. *mongolica* and A. *mono* under elevated CO<sub>2</sub>, with a decrease in the ratio of soluble N to structural N in mature leaves, which might be involved in the photosynthetic downregulation and the increased LMA. To test this hypothesis, we evaluated the effects of elevated CO<sub>2</sub> and soil N deficiency on the photosynthetic activities, leaf morphology, plant growth, and NRE during senescence in Q. *mongolica* and *A. mono*.

#### 2. Materials and Methods

# 2.1. Plant Material

One-year-old seedlings of *Q. mongolica* var. *grosseserrata* Blume and *A. mono* Maxim. var. *glabrum* (Lév. Et Van't.) Hara were obtained from Oji Forestry and Landscaping, Sapporo, Japan, and transplanted individually into free-draining 5 L plastic pots (diameter: 21 cm) filled with 1:1 (v/v) Kanuma pumice and clay loam. Each pot was placed in a tray to prevent nutrient drainage. The initial height of seedlings was 14–18 cm in both species. These species are representative deciduous broadleaved trees of the boreal forests of Northeast Asia; seedlings and adult trees have similar sensitivities to environmental stress [61]. *Quercus mongolica* is a mid-successional species and a major constituent of mature mixed forests, whereas *A. mono* is a late-successional species capable of persisting in the forest understory for most of its life. Although *Q. mongolica* expresses the same flushing-type phenology as *A. mono*, this species often shows additional shoot elongation when the environment is favorable for growth [62,63]. *Quercus mongolica* is distributed in convex terrain and on south-facing slopes where it tends to dry out [64] and often forms secondary forests even in poor soil conditions [65]. On the other hand, *A. mono* is distributed in relatively wet and fertile areas [66].

### 2.2. Elevated CO<sub>2</sub> and Soil N Supply Treatments

Seedlings were placed in a natural daylight phytotron (Koito Industries, Yokohama, Japan) equipped with CO<sub>2</sub> concentration controllers (DAIWA Air Co. Ltd., Sapporo, Japan) at Hokkaido Research Center, FFPRI, Sapporo, Japan (43° N, 141° E; 180 m a.s.l.). They were grown with  $CO_2$  at 36 Pa (ambient  $CO_2$  treatment; it was ambient  $CO_2$  at the time of this study) or 72 Pa (elevated  $CO_2$  treatment) from mid-May 2001. We set a double  $CO_2$  concentration (at that time) as the value corresponding to the  $CO_2$  concentration of RCP 6, a scenario with relatively high greenhouse gas emissions [2,3]. Each CO<sub>2</sub> treatment was replicated in two chambers; details of the  $CO_2$  treatments were described in previous studies [59,67]. Twenty-four seedlings per species were grown in each chamber; 12 seedlings were supplied with N at 52.5 mg N pot<sup>-1</sup> week<sup>-1</sup> (high-N treatment), while the other twelve received 5.25 mg N pot<sup>-1</sup> week<sup>-1</sup> (low-N treatment) in 0.5  $\times$  Hoagland solution [68] (containing 3 mM KNO<sub>3</sub>, 2 mM Ca(NO<sub>3</sub>)<sub>2</sub>/4 H<sub>2</sub>O, 0.5 mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 2.25 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.45 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM MgSO<sub>4</sub>/7 H<sub>2</sub>O, 25 μM EDTA-Fe, 4.5 µM MnCl<sub>2</sub>/4 H<sub>2</sub>O, 23 µM H<sub>3</sub>BO<sub>3</sub>, 0.4 µM ZnSO<sub>4</sub>/7 H<sub>2</sub>O, 0.15 µM CuSO<sub>4</sub>/5 H<sub>2</sub>O, and  $0.007 \ \mu M \ (NH_4)_6 Mo_7 O_{24} / 4 H_2 O)$ . In the low-N treatment, KCl, CaCl<sub>2</sub>, and KH<sub>2</sub>PO<sub>4</sub> were added to provide the same concentrations of  $K^+$  and  $Ca^{2+}$  as those in the high-N tests. Air temperature was maintained at 26/16 °C (day/night) until August and gradually decreased from 20/10 to 14/10 °C from September to November. Pots were kept in trays with water to avoid desiccation.

#### 2.3. Growth and Biomass Allocation

In order to obtain data of green leaves for calculating NRE along with examining growth responses to  $CO_2$  and N, after 100 days of treatment (24 August 2001), the dry masses of leaves, shoots, stems, coarse roots (>2 mm), and fine roots (<2 mm) of six

seedlings in each treatment group were determined after drying at 80 °C. The N content of each tissue was determined using a combustion method and an NC analyzer (Sumigraph NC-800; Sumika Chem. Anal. Service, Osaka, Japan). N content of leaves was analyzed separately by elongation order. In addition, total leaf area (LA) was measured, and the leaf area ratio (LAR; LA per whole plant biomass) and top-to-root ratio (T/R ratio; aboveground biomass per root biomass) of each seedling were calculated. Twenty-five seedlings were harvested before treatment to determine the initial mass and RGR (total-W-RGR) of plants. Total-W-RGR for 100 days was calculated using the following equation:

$$Total-W-RGR = \ln(W_{initial}) - \ln(W_{100dav}),$$
(1)

where  $W_{initial}$  (g) and  $W_{100day}$  (g) represent the initial whole plant weight and 100-day whole plant weight of each plant harvested after 100 days of treatment, respectively.

#### 2.4. Gas Exchange Measurements

Leaf gas exchange was measured in five to seven mature leaves (about 1 month old) from plants in each treatment group using an open gas exchange system (LI-6400; Li-Cor Inc., Lincoln, NE, USA). Both A. mono and Q. mongolica started leaf opening soon after the start of treatment (mid-May). A. mono exhibited secondary elongation from early June, but most individuals did not show tertiary elongation. Q. mongolica exhibited secondary elongation from mid-June and tertiary elongation from late July. However, few individuals in low N exhibited tertiary elongation. Leaf length reached its maximum by about 2 weeks regardless of tree species or treatment, and CO<sub>2</sub> and N treatments had no effect on leaf opening rates (data not shown). Mature leaves were measured on days 57-65 and 50-52 for Q. mongolica and A. mono, respectively, with each treatment. Secondary flushed leaves, which were produced under the conditions of each treatment, were used for analyses. For Q. mongolica, we also measured photosynthesis in an additional six to nine mature leaves (secondary elongation leaves aged about 1.5 months) in each treatment group during the tertiary elongation. Light-saturated net photosynthetic rates per LA ( $A_{area}$ ) were measured at both  $CO_2$  conditions (36 and 72 Pa  $CO_2$ ) for the immature leaves (about 2 weeks old; data not shown). Full  $A_{\text{area}}$  versus internal CO<sub>2</sub> ( $C_i$ ) curves were determined for mature leaves. Saturating photon flux density at the upper leaf surface was 1200  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, which was determined based on light-response curves of photosynthetic rates (data not shown). Each curve consisted of the following seven steps: 2000, 1500, 720, 360, 200, 100, and 50 µmol of  $CO_2$  mol<sup>-1</sup>. Leaf temperature was maintained at 25 °C, and the leaf-to-air vapor pressure saturation deficit was maintained below 1.2 kPa. The  $V_{cmax}$  and the maximum electron transport rate  $(J_{max})$  were calculated with the 'fitaci' function ('plantecophys' package, R version 4.0.3. [69]) proposed by Duursma [70], which uses the Farquhar, von Caemmerer, and Berry model [71]. The coefficients of temperature dependence of  $V_{\rm cmax}$  and  $J_{\rm max}$  were taken from the values of *Quercus* and *Acer* species in Dreyer [72].

# 2.5. Leaf Characters

After measuring gas exchange, leaf area was measured using an LA meter (LI-3000A; Li-Cor Inc.), and soil plant analysis development (SPAD) values, an indicator of chlorophyll content, were also measured (SPAD 502, MINOLTA, Osaka, Japan). LMA was measured after drying at 80 °C for 48 h, and then the  $N_{mass}$  was determined using the NC analyzer mentioned above. In this paper, LMA was used as an indicator of structural N. On the other hand, since LMA is also affected by contents other than N, we measured total nonstructural carbohydrate (TNC), which affects the photosynthetic response to elevated CO<sub>2</sub>, as one of them. Soluble sugars and starch contents were measured, and TNC was calculated as the sum of these contents. Using a grounded leaf sample, soluble sugars were extracted with 80% ethanol and then determined via the phenol–sulfuric acid method [73]. Starch in the residue was solubilized by potassium hydroxide and then digested to glucose with an amyloglucosidase (A9228; Sigma-Aldrich, St. Louis, MO, USA) solution. The digested

glucose was determined with the Wako Auto Kit Glucose (439–90901; Wako Pure Chemical Industries Ltd., Osaka, Japan).

Nitrogen allocation within leaves was assumed to be one of the factors influencing NRE, and the results of gas exchange measurements were used for indirect estimation of leaf N partitioning within leaves. The model proposed by Niinemets and Tenhunen [74] was used to determine the coefficient for leaf N partitioning to carboxylation capacity (mainly Rubisco;  $F_r$ : g N in Rubisco (g total leaf N)<sup>-1</sup>) and the coefficient of allocation of leaf N to energy transfer (i.e., bioenergetic pools;  $F_b$ : g N in cytochrome f, ferredoxin NADP reductase, and coupling factor (g total leaf N)<sup>-1</sup>).  $F_r$  and  $F_b$  were calculated using the follow equations:

$$F_{\rm r} = V_{\rm cmax} / (6.25 V_{\rm cr} \, \rm LMA \, N_{\rm mass}), \tag{2}$$

$$F_{\rm b} = J_{\rm max} / (8.06 J_{\rm mc} \, {\rm LMA} \, N_{\rm mass}),$$
 (3)

where  $V_{cr}$  is the specific activity of Rubisco: for 20.5 µmol CO<sub>2</sub> (g Rubisco)<sup>-1</sup> s<sup>-1</sup> (25 °C) [75], 6.25 (g Rubisco (g N in Rubisco)<sup>-1</sup>) converts *N* content to protein content; and  $J_{mc}$  is the photosynthetic electron transfer capacity per unit of cytochrome f: for 156 mol e<sup>-</sup> (mol cyt f)<sup>-1</sup> s<sup>-1</sup> (25 °C), 8.06 (µmol cyt f.) converts *N* content to energy transfer [76]. Although Frak et al. [77] suggested that this method possibly leads to underestimation, we used these parameters as indicators for the allocation ratio of N to Rubisco (e.g., [78]).

## 2.6. N Resorption Efficiency before Senescence

Leaf litter from each individual, which was remaining after 100 days sampling, was collected daily as leaves abscised from October to November. The onset of defoliation began in mid-October and ended in early to mid-November for both tree species. LMA and  $N_{\text{mass}}$  were determined in each senescent leaf as well as in leaves of the harvested seedlings after 100 days of treatment. Leaf litter data were calculated for each individual plant. NRE was calculated using the following equation [30,79–81]:

$$NRE = 100 (N_{\rm g} - N_{\rm s})/N_{\rm g},$$
 (4)

where  $N_g$  represents the average N in the green leaves of each plant harvested after 100 days of treatment, and  $N_s$  represents the average N in senescent leaves. We calculated the NRE both on a mass basis ( $NRE_{mass}$ ) and on an area basis ( $NRE_{area}$ ) because leaf weight had decreased at the time of leaf shedding due to the resorption of mobile carbohydrates and nutrients [30,79]. Recent studies on mass-based NRE used a mass loss correction factor, which was determined from leaf mass loss during senescence, to prevent nutrient resorption underestimation [36,38].  $NRE_{area}$  in this study corresponds to the corrected mass-based NRE value. Leaching of N from leaves was assumed to be minimal [82].

### 2.7. Statistical Analysis

Two-way ANOVA for split-plot designs was used to evaluate the effects of CO<sub>2</sub> and N treatments on gas exchange ( $V_{cmax}$  and  $J_{max}$ ), growth properties, green leaf characteristics, fallen leaf characteristics, and NRE; the probability level was set at p < 0.05, and analyses were conducted in R version 4.0.3 [69]. A linear mixed model was applied to analyze the  $V_{cmax}$  and  $J_{max}$ , with  $N_{area}$ , CO<sub>2</sub>, species, and the interaction of CO<sub>2</sub> and species as fixed factors, and the chamber as a random effect. A linear mixed model was also applied to analyze the  $NRE_{area}$ ,  $NRE_{mass}$ , LMA, and  $F_r + F_b$  with CO<sub>2</sub>, N, species, and their interactions as fixed factors, and the chamber as a random effect. We used the lmer function of the R package lme4, and the ANOVA function of the R package car.

#### 3. Results

### 3.1. Growth Responses of Q. mongolica and A. mono Seedlings to CO<sub>2</sub> and N Treatments

The 100-day relative growth rate of the whole plant weight (total-W-RGR) showed significant effects of CO<sub>2</sub> (p < 0.05) and N treatments (p < 0.001) in *Q. mongolica*, and an enhanced whole plant biomass was observed under elevated CO<sub>2</sub> and under high N

(Table S1). However, the total-W-RGR of *A. mono* was not influenced by both CO<sub>2</sub> and N treatment. In *Q. mongolica*, elevated CO<sub>2</sub> had a negative effect on LAR (p < 0.05). N treatments also had an effect on LAR in *Q. mongolica* and *A. mono*, with LAR in *Q. mongolica* and in *A. mono* decreasing (p < 0.01) and increasing (p < 0.05) at low N, respectively. The T/R ratio was not influenced by CO<sub>2</sub> treatments in either species, but it decreased under low N in *Q. mongolica* (p < 0.001).

# 3.2. Maximum Carbon Fixation Rate ( $V_{cmax}$ ) and Maximum Electron Transfer Rate ( $J_{max}$ ) of Q. mongolica and A. mono Seedlings under CO<sub>2</sub> and N Treatments

In A. mono (1-month-old leaves), both the  $V_{cmax}$  and  $J_{max}$  showed an effect of  $CO_2$  treatment and decreased under elevated  $CO_2$  compared with these rates under ambient  $CO_2$  conditions, but they showed no effect of N treatments (Figure 1B,D). In 1-month-old leaves of Q. mongolica,  $J_{max}$  was not affected by  $CO_2$  treatment, and only  $V_{cmax}$  was decreased by elevated  $CO_2$  and low N (data not shown). On the other hand, in 1.5-month-old leaves of Q. mongolica, both  $V_{cmax}$  and  $J_{max}$  were reduced by elevated  $CO_2$  and low N, but  $V_{cmax}$  showed an interactive effect of N and  $CO_2$  treatments (Figure 1A,C). These results indicate that the leaf age, which clearly showed the downregulation of photosynthesis, was different between A. mono (1-month-old leaves) and Q. mongolica (1.5-month-old leaves).



**Figure 1.** Maximum carbon fixation rate ( $V_{cmax}$ ) and maximum electron transfer rate ( $J_{max}$ ) of *Quercus mongolica* (1.5-month-old leaves) (**A**,**C**), and *Acer mono* (**B**,**D**). Means and standard errors are shown (n = 5-9). Ambient CO<sub>2</sub>: 36 Pa; elevated CO<sub>2</sub>: 72 Pa. *F*-value and probability (p) of the effects of CO<sub>2</sub> ( $p_c$ ), N ( $p_N$ ), and their interaction ( $p_{CxN}$ ) are indicated in the panel. Lowercase letters represent multiple comparison results among four treatments when the treatment effects are significant at p < 0.05.

# 3.3. Characteristics of Green Mature Leaves of Q. mongolica and A. mono Seedlings under CO<sub>2</sub> and N Treatments

In *Q. mongolica* (1.5-month-old leaves) and *A. mono* (1-month-old leaves), the  $N_{mass}$  of leaves decreased with high CO<sub>2</sub> (p < 0.01 and p < 0.05, respectively) and low N (p < 0.001 and p < 0.01, respectively), whereas LMA increased under elevated CO<sub>2</sub> compared to under ambient CO<sub>2</sub>, regardless of N treatments (p < 0.001 and p < 0.01, respectively; Table 1). As a result, in *Q. mongolica* and *A. mono*, the  $N_{area}$  decreased under low N compared to under high N (p < 0.001 for both). However, no significant effect of CO<sub>2</sub> treatment on  $N_{area}$  (p > 0.05) was observed. SPAD values decreased in *Q. mongolica* under low N (p < 0.001) and in *A. mono* under elevated CO<sub>2</sub> (p < 0.05). The concentration of TNC in mature

leaves increased significantly with elevated  $CO_2$  in *Q. mongolica* (1.5-month-old leaves, p < 0.05) and in *A. mono* (p < 0.01). There was no significant change in the soluble sugar concentration, and the increase in the starch concentration corresponded to the change in the TNC concentration. In *Q. mongolica*, the TNC concentration was higher in the low-N treatment than in the high-N treatment. The characteristics of the 1-month-old leaves of *Q. mongolica* differed from those of the 1.5-month-old leaves only in the fact that the TNC was not affected by the  $CO_2$  and N treatments.

**Table 1.** Leaf mass per area (LMA), mass-based leaf N ( $N_{mass}$ ), area-based leaf N ( $N_{area}$ ), soil plant analysis development (SPAD) values, and total nonstructural carbohydrate (TNC; sum of soluble sugar and starch) according to CO<sub>2</sub> and N treatments in *Quercus mongolica* (*Quercus*; 1.5-month-old leaves) and *Acer mono* (*Acer*; 1-month-old leaves). Mean values (standard error) for each treatment are shown (n = 5-9). Results of ANOVA (*F*-value) for CO<sub>2</sub> treatments (ambient: ambient: CO<sub>2</sub>; elevated: elevated CO<sub>2</sub>) and N treatments (high N, low N) are shown. \* p < 0.05; \*\*\* p < 0.01; \*\*\* p < 0.001.

Characteristics	Species	High N		Low N		Source of Variance		
		Ambient	Elevated	Ambient	Elevated	CO <sub>2</sub>	Ν	$\textbf{CO}_2 \times \textbf{N}$
LMA	Quercus	64.3 (1.7)	71.3 (2.1)	63.4 (1.7)	78.9 (2.8)	25.3 ***	2.9	4.2
(g m <sup>-2</sup> )	Acer	42.7 (1.2)	51.2 (2.6)	41.2 (1.5)	48.3 (2.8)	11.8 **	1.0	0.09
$N_{ m mass}$ (mg g <sup>-1</sup> )	Quercus	29.8 (1.3)	23.6 (1.4)	10.8 (0.4)	8.4 (0.3)	12.8 **	286.0 ***	3.6
	Acer	26.1 (2.0)	21.7 (1.1)	20.6 (1.5)	17.0 (1.2)	7.9 *	13.3 **	0.09
N <sub>area</sub>	Quercus	1.9 (0.10)	1.7 (0.10)	0.68 (0.03)	0.66 (0.04)	1.2	192.8 ***	2.1
(g m <sup>-2</sup> )	Acer	1.1 (0.09)	1.1 (0.03)	0.85 (0.08)	0.81 (0.05)	0.17	18.4 ***	0.04
SPAD value	Quercus	42.0 (1.3)	41.9 (1.4)	21.1 (1.6)	20.8 (1.0)	0.73	247.8 ***	0.003
	Acer	33.1 (0.6)	29.7 (1.1)	30.4 (1.7)	27.8 (1.4)	5.4 *	3.3	0.13
TNC	Quercus	7.6 (1.3)	12.6 (2.7)	15.4 (1.5)	20.1 (1.6)	4.9 *	19.4 ***	0.004
(%)	Acer	7.8 (1.4)	13.1 (2.6)	7.5 (1.4)	14.3 (2.8)	10.6 **	0.06	0.15

3.4. Indirect Estimation of N Partitioning within Leaves of Q. mongolica and A. mono Seedlings under CO<sub>2</sub> and N Treatments

When the two N treatments were analyzed together, the relationship between the  $N_{\text{area}}$ and  $V_{\rm cmax}$  of Q. mongolica (1.5-month-old leaves) and A. mono (1-month-old leaves) was affected by CO<sub>2</sub> treatment but not by species, and  $V_{cmax}$  for  $N_{area}$  decreased 10.6 µmol m<sup>-2</sup> s<sup>-1</sup> under elevated CO<sub>2</sub> compared with under ambient CO<sub>2</sub> (Figure 2A,B). The relationship between  $N_{\text{area}}$  and  $J_{\text{max}}$  was affected by CO<sub>2</sub> treatment and by species, and  $J_{\text{max}}$  for  $N_{\text{area}}$ decreased by 17.1  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> under elevated CO<sub>2</sub> and was 17.3  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> lower in Q. mongolica than in A. mono (Figure 2C,D). The partitioning of N to the photosynthetic function within leaves, i.e.,  $F_r$  and  $F_b$ , which were estimated from  $V_{cmax}$  and  $J_{max}$ , was affected by CO<sub>2</sub> treatment in both species and by N treatment only in A. mono (Table 2).  $F_r$  and  $F_b$  were significantly decreased by elevated CO<sub>2</sub> in *Q. mongolica* (1.5-month-old) and in A. mono and were increased under low N in A. mono. The characteristics of the 1-month-old leaves of Q. mongolica differed from those of the 1.5-month-old leaves only in the fact that  $F_{\rm b}$  was not affected by the CO<sub>2</sub> treatments. The total ratio of N allocation to the photosynthetic function, i.e.,  $F_r + F_b$ , showed similar responses to the CO<sub>2</sub> and N treatments to each parameter ( $F_r$ ,  $F_b$ ), for each species. Photosynthetic N use efficiency (PNUE) to  $C_i$ decreased under elevated  $CO_2$  and increased under low N in A. mono (ANOVA; F = 8.4, p < 0.01 and F = 7.5, p < 0.05, respectively), and it decreased under elevated CO<sub>2</sub> in the older (1.5-month-old) leaves of *Q. mongolica* (ANOVA; F = 17.5, p < 0.001).



**Figure 2.** Relationship between area-based leaf N content ( $N_{area}$ ) and maximum carbon fixation rate ( $V_{cmax}$ ) and maximum electron transfer rate ( $J_{max}$ ) of *Quercus mongolica* (1.5-month-old leaves) (**A**,**C**) and *Acer mono* (1-month-old leaves) (**B**,**D**). Open symbols: ambient CO<sub>2</sub>; closed symbols: elevated CO<sub>2</sub>; circles: high N; triangles: low N. Results of the generalized linear mixed model ( $\chi^2$ -value and *p*-value) are indicated besides the panel. The two N treatments (high N and low N) were analyzed together in this instance.

**Table 2.** The allocation coefficient of leaf N to carboxylase reactions ( $F_r$ : g N in Rubisco (g total leaf N)<sup>-1</sup>) and to bioenergetic pools ( $F_b$ : g N in cytochrome f, ferredoxin NADP reductase, and coupling factor (g total leaf N)<sup>-1</sup>) in *Quercus mongolica* (*Quercus*; 1.5-month-old leaves) and *Acer mono* (*Acer*; 1-month-old leaves). Mean values (standard error) for each treatment are shown (n = 5–9). Results of ANOVA (*F*-value) for CO<sub>2</sub> treatments (ambient: ambient CO<sub>2</sub>; elevated: elevated CO<sub>2</sub>) and N treatments (high N, low N) are shown. \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001.

Characteristics	Species	High N		Low N		Source of Variance		
		Ambient	Elevated	Ambient	Elevated	CO <sub>2</sub>	Ν	$\textbf{CO_2} \times \textbf{N}$
Fr	Quercus Acer	0.29 (0.01) 0.30 (0.01)	0.26 (0.01) 0.22 (0.02)	0.33 (0.01) 0.40 (0.02)	0.26 (0.02) 0.31 (0.03)	17.2 *** 17.6 ***	1.3 18.8 ***	1.1 0.005
Fb	Quercus	0.050 (0.002)	0.047 (0.002)	0.054 (0.002)	0.051 (0.003)	4.4 *	2.6	0.12
	Acer	0.066 (0.004)	0.048 (0.003)	0.074 (0.003)	0.065 (0.005)	12.0 **	10.2 **	1.3

3.5. N Resorption Efficiency of Q. mongolica and A. mono Seedlings under CO<sub>2</sub> and N Treatments

In both species,  $NRE_{area}$  and  $NRE_{mass}$  decreased under elevated CO<sub>2</sub> levels relative to  $NRE_{area}$  and  $NRE_{mass}$  under ambient CO<sub>2</sub> conditions, respectively (Figure 3A,B). The responses of  $NRE_{area}$  and  $NRE_{mass}$  to N treatment differed in the two species. The  $NRE_{area}$ of *Q. mongolica* was affected by CO<sub>2</sub> and N treatments, with a decrease under elevated CO<sub>2</sub> compared to ambient CO<sub>2</sub> (-18.7%) and under low N compared to high N (-6.3%; Figure 3A). The  $NRE_{area}$  of *A. mono* was also affected by CO<sub>2</sub> and N treatments, with a decrease under elevated CO<sub>2</sub> (-7.7%), but, unlike *Q. mongolica*, it increased under low N (+11.4%). The  $NRE_{mass}$  of *Q. mongolica* also significantly decreased under elevated CO<sub>2</sub> (-21.8%) and under low N (-8.9%; Figure 3B). The  $NRE_{mass}$  of *A. mono* showed interactive effects for CO<sub>2</sub> and N treatments, with a tendency to decrease under elevated CO<sub>2</sub> (-9.0%) and to increase under low N (+14.7%). The LMA of senesced leaves increased with elevated CO<sub>2</sub> in both species, regardless of the N treatment (Figure 3E). The  $N_{mass}$  of senesced leaves in *Q. mongolica* showed interactive effects for CO<sub>2</sub> and N treatments, with a tendency to increase under elevated CO<sub>2</sub>, and to decrease under low N (Figure 3D). Meanwhile, the  $N_{\text{mass}}$  of *A. mono* was not significantly affected by CO<sub>2</sub> treatment but was affected by N treatment, with a decrease under low-N conditions. The  $N_{\text{area}}$  of the senesced leaves showed similar responses to those of  $N_{\text{mass}}$  (Figure 3C). The C/N ratio of senesced leaves decreased with elevated CO<sub>2</sub> in *Q. mongolica* and increased with low N in both species (Figure 3F).



**Figure 3.** Area-based N resorption efficiency (NRE) (*NRE*<sub>area</sub>; (**A**)) and mass-based NRE (*NRE*<sub>mass</sub>; (**B**)) during defoliation as well as area-based N content ( $N_{area}$ ; (**C**)), mass-based N content ( $N_{mass}$ ; (**D**)), leaf mass per area (LMA; (**E**)), and C/N ratio (**F**) of fallen leaves of *Quercus mongolica* and *Acer mono*. Means and standard errors are shown (n = 6). Open bars: *Q. mongolica*; closed bars: *A. mono*; ambient CO<sub>2</sub>: 36 Pa; elevated CO<sub>2</sub>: 72 Pa. *F*-value and probability (*P*) of the effects of CO<sub>2</sub> ( $p_C$ ), N ( $p_N$ ), and their interaction ( $p_{CxN}$ ) for *Q. mongolica* (*Quercus*) and *A. mono* (*Acer*) are indicated in or above the panel. Uppercase and lowercase letters represent multiple comparison results among four treatments when the treatment effects are significant at p < 0.05 for *Q. mongolica* and *A. mono*, respectively.

### 4. Discussion

4.1. N Content in Senesced Leaves of *Q.* mongolica and *A.* mono Seedlings Showed No Decline under Elevated CO<sub>2</sub> Regardless of N Conditions

Elevated  $CO_2$  can change leaf litter biomass and chemistry (N content, C/N ratio, lignin/N ratio, etc.), which can, in turn, affect the decomposition rate of leaf litter, e.g., by

microbiology [13,26,83], and may also influence the availability of N that can be absorbed by trees [46,84,85]. Many tree species have been reported to show decreased N concentrations in green leaves under elevated CO<sub>2</sub> conditions [13,14]. In our study, the  $N_{\text{mass}}$  of senesced leaves decreased under low N in both species and showed an increase (Q. mongolica) or no effect (A. mono) by elevated CO<sub>2</sub>. As a result, the C/N ratio of senesced leaves did not increase with elevated CO<sub>2</sub> in both species, though it increased with low N in both species. These results support the finding that leaf litter N content shows little or no decline under elevated CO<sub>2</sub> [86] but do not support the litter quality hypothesis [18]. Similarly, elevated CO<sub>2</sub> has little or no effect on the N content in the leaf litter of *Quercus rubra* [21]. On the other hand, *Acer rubrum* [22,23] and *Tilia americana* [47] show a decline in N in senescent leaves under elevated CO<sub>2</sub> conditions. Therefore, the question remains, why does high CO<sub>2</sub> have no clear effect on the  $N_{\text{mass}}$  of leaf litter while tending to cause  $N_{\text{mass}}$  to decrease in green leaves in our study?

### 4.2. N Resorption Efficiency of Q. mongolica and A. mono Seedlings under CO<sub>2</sub> and N Treatments

Nutrient resorption varies greatly among plant species, functional types [36,37,87], plant age [33,34], and numerous abiotic factors, such as soil N availability [30,31], N additions [32], and climate zone (mean annual precipitation and latitude). Nutrient resorption can both increase [22,23,43,46,47] and decrease [16,39–41] under increasing CO<sub>2</sub> conditions. Furthermore, it has been reported that elevated  $CO_2$  had no effect on N resorption in Quercus rubra [21] and other species [22,42–45,88]. In the present study, NRE was evaluated as both per leaf mass and per leaf area; the NRE during defoliation of Q. mongolica and A. mono decreased under elevated CO<sub>2</sub> in both cases, though in A. mono, the NRE<sub>mass</sub> displayed interactive effects of CO2 and N. During defoliation, leaf weight loss (mass resorption) occurs and LMA decreases; hence, it is desirable to evaluate NRE per unit leaf area [30,36,38,89]. In the present study, NRE<sub>area</sub> was 5.3% higher in A. mono and 4.6% higher in *Q. mongolica* than  $NRE_{mass}$ , although there was an interactive effect between N and CO<sub>2</sub> treatment and the assessment method in *Q. mongolica* (GLMM, p < 0.05). The *NRE*<sub>area</sub> of *Q*. mongolica ranged from 62% to 81%, and A. mono ranged from 48% to 67%, both of which are equal to or higher than the range of values reported for several species [23,24,37,87,90]. It has been well documented that NRE is higher under poor nutritional status [30,31], and that A. mono also had higher NRE at low N, independent of CO<sub>2</sub> conditions. Lower NRE values during defoliation under high  $CO_2$  may have obscured the effect of high  $CO_2$  on the  $N_{\text{mass}}$  of senescent leaves, despite the  $N_{\text{mass}}$  of green leaves showing a downward trend under high CO2. Another question therefore arises: why did NRE decrease under high CO<sub>2</sub> condition?

# 4.3. Relationship between NRE and N Partitioning within Leaves of Q. mongolica and A. mono Seedlings under $CO_2$ and N Treatments

N is distributed in leaves among structural and metabolic proteins [55,91]; the ratio of soluble to structural N is considered one of the most important factors controlling NRE [40]. N allocated to structural proteins is used, for example, in cell walls and is not easily reabsorbed [49,91–93]. LMA correlates not only with the thickness [94] and amount of material [95,96] in the cell wall but also with the amount of intraleaf N allocated to structural proteins [55]. In mature leaves, LMA often increases under high CO<sub>2</sub> [51]; this was also shown in our study, in which LMA increased in mature leaves under elevated CO<sub>2</sub> in both *Q. mongolica* (+6.7 g m<sup>-2</sup>) and *A. mono* (+8.5 g m<sup>-2</sup>). Thus, we infer that the N allocation to structural proteins may also be larger with increased CO<sub>2</sub> in both species. However, the increase in LMA was also influenced by the increase in starch under elevated CO<sub>2</sub>, and in our study, area-based TNC increased by +4.1 g m<sup>-2</sup> in *Q. mongolica* and +3.7 g m<sup>-2</sup> in *A. mono* under elevated CO<sub>2</sub> conditions. These results indicate that an increase in LMA does not necessarily mean that structural N increased in mature leaves.

N allocated to metabolic proteins, many of which are enzymes associated with photosynthesis [48], is easily degraded and reabsorbed during leaf senescence [49,50]. The most abundant plant protein is Rubisco, an enzyme of the photosynthetic system, which accounts for 15-30% of all proteins in C<sub>3</sub> plant leaves [48,76]. Downregulation of photosynthesis under high CO<sub>2</sub> often leads to a decrease in the amount of Rubisco or in  $V_{cmax}$ , which represents the activity of Rubisco [51–53]. In our study, both  $V_{\text{cmax}}$  and  $J_{\text{max}}$  decreased under high CO<sub>2</sub> in mature Q. mongolica (1.5-month-old) and A. mono (1-month-old) leaves. In addition, the sum of the allocation ratios  $(F_r, F_b)$  of the N fractions to the Rubisco and the electron transfer system also decreased significantly in both species under high CO<sub>2</sub> levels. These results imply a decrease in the allocation of N to metabolic proteins under elevated  $CO_2$  and suggest that this decrease influences the decrease in NRE at high  $CO_2$  in the mature leaves of both species. A similar relationship between changes in the N allocation ratio to metabolic proteins and NRE is suggested by the fact that the sum of the allocation ratios (F<sub>r</sub>, F<sub>b</sub>) and NRE in mature A. mono leaves increased in low-N conditions. The PNUE has been suggested to be relatively low in tree species with higher allocations to structural non-photosynthetic elements in leaf N [97]. The reduction in the N allocation to metabolic proteins under high  $CO_2$  may also be suggested by the reduced PNUE (without increased SPAD) response of both species under elevated  $CO_2$  conditions. Overall, these results suggest that the photosynthetic response to high CO<sub>2</sub> and low N affects NRE through N allocation in the leaves. When the sum of the N allocation ratio,  $F_r + F_b$ , was used as an index of soluble (metabolic) N, and LMA was used as an index of structural N, the results support the hypothesis that NRE may decrease through changes in the leaf ratio of soluble N to structural N under high CO<sub>2</sub> [16,39–41] in both *Q. mongolica* and *A. mono*.

# 4.4. Response of NRE with $N_2$ Fixer and Non- $N_2$ Fixer (Q. mongolica and A. mono) to $CO_2$ and N Treatments

The photosynthetic activity and growth responses of  $N_2$  fixers to increased CO<sub>2</sub> may be more direct than those of non-N<sub>2</sub> fixers [52,57,58]. The NRE of N<sub>2</sub> fixers under ambient  $CO_2$  conditions is usually lower than that of non-N<sub>2</sub> fixers [36,38]. We previously reported that in an N<sub>2</sub> fixer, Alnus hirsuta, NRE<sub>mass</sub> was not affected by elevated CO<sub>2</sub>, regardless of N conditions, while  $NRE_{\text{area}}$  tended to decrease under elevated CO<sub>2</sub> with low N levels [59]. Moreover, only V<sub>cmax</sub> for N<sub>area</sub> decreased with elevated CO<sub>2</sub> in A. hirsuta (Figure S1), and, in the allocation ratios ( $F_r$ ,  $F_b$ ) of the N fractions within the leaves, only  $F_r$  decreased with elevated CO<sub>2</sub> in A. hirsuta (Table S2), with the sum of  $F_r$  and  $F_b$  showing no effect of the elevated  $CO_2$ . These results also suggest that there may be an association between the lower reduction in the N allocation to metabolic proteins at high  $CO_2$  and the unclear response in NRE. In A. hirsuta, the increase in LMA of mature leaves was also small under elevated  $CO_2$  [59,60]. Overall, the allocation of N to metabolic system proteins under high  $CO_2$ seems to be largely reduced in Q. mongolica and A. mono compared with A. hirsuta under the same conditions. The two non-N<sub>2</sub> fixers, Q. mongolica and A. mono, may be more prone to photosynthetic downregulation under high CO<sub>2</sub> than the N<sub>2</sub> fixer A. hirsuta, resulting in lower N allocation ratios to metabolic proteins within leaves and a distinct decrease in NRE at elevated  $CO_2$  conditions. We speculate that these interspecific differences in N allocation responses to elevated  $CO_2$  may affect not only the photosynthetic response of mature leaves but also the changes in NRE.

### 4.5. Phtosynthetic Responses of Q. mongolica and A. mono Seedlings under $CO_2$ and N Treatments

In the present study, the magnitude of the decrease in photosynthetic activity under elevated  $CO_2$  differed among leaf maturation stages and between tree species. In *Q. mongolica* and *A. mono*, downregulation of photosynthesis occurred earlier in *A. mono* (in 1-month-old leaves) than in *Q. mongolica* (in 1.5-month-old leaves). Furthermore, *A. mono* showed a decrease in photosynthetic capacity even with high N levels, suggesting that N deficiency was not the only cause of the decreased photosynthetic capacity with high  $CO_2$  levels. Since a genetic limitation of leaf opening, such as a determinant growth pattern, can accelerate the downregulation of photosynthesis [51,52], we considered the possibility that the sink limit was caused by the simultaneous leaf opening nature of *A. mono*. It is possible that the plasticity of the leaf opening style of *Q. mongolica*, which is more prone to tertiary elongation under different growth conditions than that of *A. mono*, led to a larger sink that

affected the high  $CO_2$  response. Since, contrary to expectations, photosynthesis was also downregulated during tertiary elongation in *Q. mongolica*, and an accumulation of starch was observed in the leaves under high  $CO_2$ , we suggest that the source–sink balance could not be regulated by tertiary elongation alone.

### 4.6. Evaluation Method of N Resorption Efficiency

When evaluating NRE, the experimental setup and sampling method can influence the results [22,92]. In our study, we used a phytotron and did not fix the position of collecting mature and fallen leaves in the same individual; therefore, it is possible that we artificially influenced the assessment of the N concentration in fallen leaves and NRE during defoliation. Killingbech [29] used a N concentration of 1% in fallen leaves as an indicator of incomplete resorption. On the other hand, Norby et al. [16] showed that the effect of high CO<sub>2</sub> was significant when the N concentration of fallen leaves exceeded 1%. In the present study, the average N concentration of leaf litter was sometimes higher than 1% in *A. mono*, and thus we cannot exclude the possibility that N resorption was incomplete. Although there is no unified view on the response of NRE to high CO<sub>2</sub>, the results of this experiment are useful for considering material cycling under high-CO<sub>2</sub> conditions.

# 5. Conclusions

The mature leaves of two non-N<sub>2</sub>-fixing deciduous broadleaved tree species, *Q. mongolica* and *A. mono*, showed clear photosynthetic downregulation under elevated CO<sub>2</sub> conditions. In addition, the range of change in the LMA of green leaves from *Q. mongolica* and *A. mono* was greater under elevated CO<sub>2</sub>. These differences in photosynthetic ( $V_{cmax}$ and  $J_{max}$ ) and morphological (LMA) responses to elevated CO<sub>2</sub> may affect NRE during defoliation via N allocation changes within leaves. Overall, these results support the hypothesis that NRE is reduced in *Q. mongolica* and *A. mono* under elevated CO<sub>2</sub>, with a decrease in the ratio of soluble N to structural N in mature leaves. In addition, these results also suggest that NRE in the non-N<sub>2</sub> fixers *Q. mongolica* and *A. mono* responds to elevated CO<sub>2</sub> in a different manner than the NRE of the N<sub>2</sub> fixer *A. hirsuta*, in part because of differences in the plants' photosynthetic activities. We speculate that these interspecific differences in N allocation responses to elevated CO<sub>2</sub> may affect not only the photosynthetic response of mature leaves but also the changes in NRE.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10 .3390/f12081034/s1: Figure S1: Relationship between area-based leaf N content ( $N_{area}$ ) and maximum carbon fixation rate ( $V_{cmax}$ ) (A) and maximum electron transfer rate ( $J_{max}$ ) (B) of *Alnus hirsuta*; Table S1: Relative growth rate of whole plant weight (total-W-RGR), leaf area ratio (LAR), leaf weight ratio (LWR), fine root ratio (fine RWR/RW), and T/R ratio in *Quercus mongolica* (*Quercus*) and *Acer mono* (*Acer*); Table S2: Allocation of the coefficient of leaf N to carboxylase reactions ( $F_r$ : g N in Rubisco (g total leaf N)<sup>-1</sup>) and to bioenergetic pools ( $F_b$ ; g N in cytochrome f, ferredoxin NADP reductase, and coupling factor (g total leaf N)<sup>-1</sup>) in *Alnus hirsuta*.

**Author Contributions:** H.T. and M.K. designed the research and collected the photosynthetic data. H.T., M.K., A.U. and H.U. performed the data analysis. H.T. led the writing. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The data presented in this study are available within the article and its Supplementary Materials.

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